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TITLE: Direct inhibition of Skp2 for the Treatment of Advanced Prostate Cancer

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## Table of Contents

|                                   | <u>Page</u> |
|-----------------------------------|-------------|
| Introduction.....                 | 4           |
| Body.....                         | 4           |
| Key Research Accomplishments..... | 7           |
| Reportable Outcomes.....          | 7           |
| Conclusion.....                   | 7           |

## INTRODUCTION

The goal of this project is to identify molecules that block the growth of late stage prostate cancer cells through the inhibition of Skp2 signaling. To this end, we performed a high-throughput screening of cyclic peptoid libraries to identify a molecule that directly binds Skp2 with low micromolar affinity. This compound was found to effectively reduce cell viability of cancerous cells. With the compelling preliminary results, further studies are now in progress to evaluate the activity of the compound as a novel class of anti-prostate cancer agents.

## BODY

Here I will describe the research accomplishments (in bold) associated with each task outlined in the approved Statement of Work.

### (Statement of Work)

Task 1 for specific aim 1: To perform high-throughput “on-bead” screening of combinatorial peptoid libraries to identify molecules that directly bind to Skp2 (3 months)

Subtask 1. Solid-phase synthesis of combinatorial “one-bead one-compound” peptoid libraries by the conventional split-and-mix method (1.5 months)

- Synthesis of cyclic and linear 5-mer peptoid libraries with diversity of 1,000,000 (Synthesis of a 5-mer linear peptoid library has already been done)

**We synthesized combinatorial libraries of both linear and cyclic peptoids (Figure 1A) as planned.**

Subtask 2. Expression/purification/biotinylation of Skp2-Skp1 complex (already done)

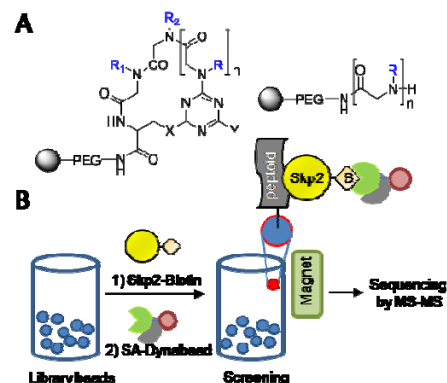
- Skp1-Skp2-GST expression in *E. coli* (BL21)
- Protein purification by several round of column (GST column before/after GST cleavage followed by gel filtration column)
- Biotinylation of the purified Skp1-Skp2 with biotin-NHS ester (Pierce)

**Skp1-Skp2 protein complex was successfully purified in high quality by FPLC purification and then biotinylated with the biotinylation kit (degree of labeling was ~ 1.2) without any problems.**

Subtask 3. High-throughput “on-bead” screen of the synthesized libraries (1.5 months)

- On-bead screen of library against Skp2-Skp1 and sequencing of “hit” beads by tandem mass spectrometry (Six putative “hit” peptoids are already in hand from a screen of the linear peptoid library)

Task 2 for specific aim 2: To validate the binding of the identified “hit” peptoids to Skp2 through biochemical assays (3.5 months)



**Figure 1.** (A) General structures of cyclic and linear peptoid libraries. (B) A protocol of “on-bead” screening (SA = streptavidin).

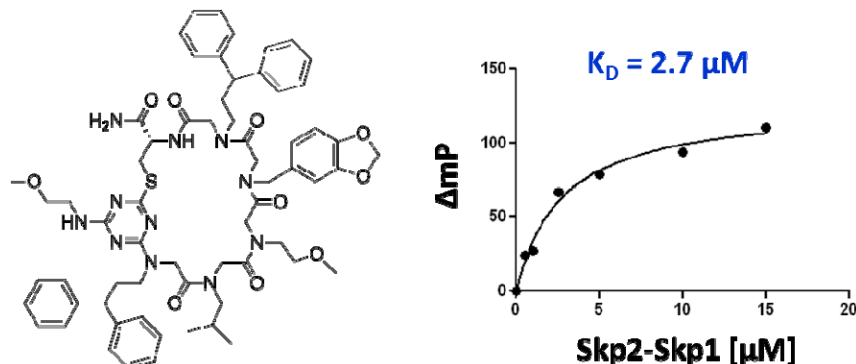
The above-mentioned six putative “hit” peptoids obtained from on-bead screening of linear peptoids were resynthesized as fluorescently-labeled forms. The binding affinity of these compounds was examined by fluorescence polarization assays. Unfortunately, their binding affinity was very weak. Therefore, we carried out another screening with cyclic peptoid library. By using cyclic peptoids which are far more conformationally rigid than linear peptoids, we envisioned that the chance of getting hit compounds should be increased.

Cyclic peptoids generated by Subtask 1 were screened for their ability to bind to Skp2-Skp1 (purified and biotinylated in Subtask 2) via on-bead screening (Figure 1B). This screening resulted in the identification of a hit compound.

Subtask 1. Fluorescence polarization assay to determine the binding affinity (1.5 months)

- Synthesis and purification of fluorescein-labeled “hit” peptoids (1 month)
- Fluorescence polarization assay (0.5 month)

The hit compound was synthesized as a fluorescence-labeled form and used in fluorescence polarization-based binding assays to determine its binding affinity to Skp1-Skp2 protein complex.



**Figure 2.** Chemical structure of the hit compound and binding affinity determined by fluorescence polarization assay.

Subtask 2. Immunoprecipitation assay to test the ability of the peptoids to inhibit protein-protein interaction of Skp2 with its binding proteins such as Cks1 and p300 (2 months)

Subtask 3. X-ray studies (3 months)

- To determine the co-crystal structures of Skp2-Skp1 in complex with the most active peptoids (the experiments will be performed in the laboratory of Prof. Thomas Hurley (a collaborator))

**For X-ray studies, we are purifying Skp2-Skp1 in a large scale. Experiments to obtain crystal structures of Skp2-Skp1 in complex with the cyclic peptoid will be started soon.**

Task 3 for specific aim 3: To determine the inhibitory efficacy of the peptoids on Skp2 signaling in advanced prostate cancer cells (5.5 months)

Subtask 1. To test the effects of the peptoids on cellular protein degradation in prostate cancer cells (1.5 month)

- After treating AR-dependent (LNCaP cells) or independent (DU-145 cells) cells with the peptoids, accumulation of p27, p21 and p57 will be accessed by immunoblot analysis.

First, Western blotting experiments were performed with HeLa cells. The cyclic peptoid indeed increased cellular levels of p27, indicating the compound is cell permeable and act as inhibitors of Skp2. Further studies with prostate cancer cells will be done soon.

Subtask 2. Real-time PCR analysis of PSA to examine the effect of the identified peptoids on AR-dependent transcription in prostate cancer cells (e.g. LNCaP) (1 month)

**This experiment will be carried out soon.**

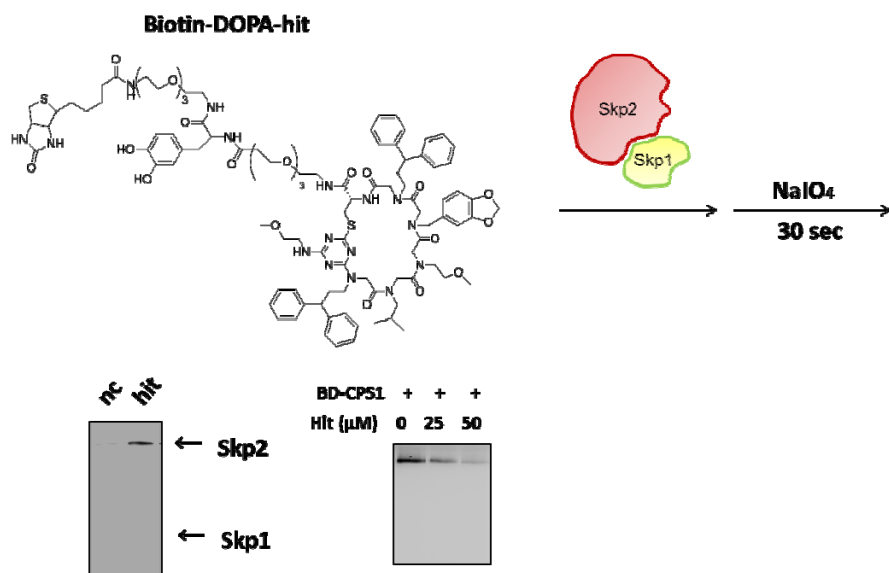
Subtask 3. To examine the effect of the peptoids on the cell cycle and apoptosis (3 months)

- Flow cytometry analysis, CellTiter-Glo (Promega) cell viability assay and caspase assay using a kit (EnzCheck caspase-3 assay kit, Invitrogen)

Several different cancerous cells including U266 leukemia cells and MCF-7 human breast cancer cells were treated with DMSO (as a negative control) and varying concentrations of the cyclic peptoid. And cell viability was monitored by CellTiter-Glo (Promega) cell viability assay kit. Indeed, the compound effectively reduced the cell viability of the cells. We will further be examined the activity of the compound as a Skp2 inhibitor by different assays with prostate cancer cells.

### Chemical cross-linking experiments

Since Skp1-Skp2 complex was employed in the screen, it was not clear whether the identified cyclic peptoid targets Skp2. To identify the molecular target of the compound, we carried out chemical cross-linking experiments. First we synthesized biotinylated and DOPA-conjugated compound. Purified Skp1-Skp2 was incubated with the compound, and then treated with NaIO<sub>4</sub> for 30 second. Gel electrophoresis followed by Western blotting clearly showed that the compound directly binds to Skp2 but not Skp1. We concluded that the compound is a Skp2 ligand.



**Figure 3.** Chemical cross-linking experiments.

## KEY RESEARCH ACCOMPLISHMENTS

- **Identification of directly-acting chemical inhibitors of Skp2 as potential anti-prostate cancer agents**

## REPORTABLE OUTCOMES

- **A published paper:** Lee JH, Kim HS, Lim HS, “Design and facile solid-phase synthesis of conformationally constrained bicyclic peptoids.” *Org. Lett.* **2011**, *13*, 5012-5015.
- **Further publication and patent filing will be made.**

## CONCLUSION

Skp2 is highly upregulated in invasive late stage prostate cancers and its expression is essential for both AR-dependent and independent tumor progression. Thus, inhibition of Skp2 is a promising and validated strategy for the treatment of prostate cancers. In this project, we have successfully identified cyclic peptoids that directly bind and inhibit Skp2. The compounds have a great potential as a novel class of anti-prostate cancer agents. Further studies are now in progress.